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PRINCIPAL INVESTIGATOR: Yan Zhang, M.D., Ph.D.

CONTRACTING ORGANIZATION: The Burnham Institute
La Jolla, California 92037

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Yan Zhang, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Burnham Institute
La Jolla, California 92037

E-Mail: yzhang2@itsa.ucsf.edu

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13. ABSTRACT (Maximum 200 Words)

The hypothesis of my project is that the behavior of an invasive tumor cell is largely determined by a complex collection of numerous proteins on the surface of metastasis cell. I proposed to create a high-density single chain variable fragments (scFvs) antibody chip that can be employed to compare and quantitate the expression levels of thousands of different plasma membrane proteins on the invasive vs. non-invasive breast carcinoma cell lines. Toward this goal, I have achieved two tasks during this period of award: 1) a competitive binding condition has been worked out to allow semi-quantification of the amount of antigen bound by individual phage-scFv clone, 2) the binding feature of well characterized eight individual phage clones to cell lysate of invasive breast carcinoma MDA-MB-435 cell line provides the initial parameter for the development fo a "prototype" chip to compare the expression level of a large amount of cell surface proteins by using a 96-well plate format.

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INTRODUCTION

Since breast cancer is a common form of cancer among women, and metastasis of the cancer to distant sites is the major cause of mortality in these women, it is vitally important to understand the mechanisms involved in a tumor's progression to the metastasis phenotype.

Due to the multifactorial and complex nature of tumor metastasis process, I proposed to develop a novel methodology for studying the tumor cell as a biological system. My study is emphasizing on the proteins at the plasma membrane of the invasive and non-invasive carcinoma cell because these proteins are likely responsible for many of the phenotypic changes that occur during the transformation of the tumor cell to one with metastatic potential. The disciplines of phage display, mass spectrometry and micro-array technologies are combined in my proposal to develop a methodology for quantifying thousands of plasma membrane proteins simultaneously and identifying a collection of target antigens. By comparing of the plasma membrane protein profiles of phenotypically distinct cell lines(invasive breast cancer cell line, non-invasive breast cancer cell line and normal mammary epithelial cell line), I will decipher the subset of proteins that are required for a tumor to progress to the metastatic phenotype.

BODY

During the course of my second funding year, my effort has focused on the aim two and aim three indicating in my fellowship proposal.

My ultimate goal is to create a high-density scFv antibody chip that can be used to measure and compare the expression levels of thousands of different proteins present on the surface of non-invasive and invasive breast carcinoma cell lines. Toward this goal, I have concentrated on two tasks during this period: 1) work out competitive binding condition that allows semi-quantification of the amount of antigen bound by individual phage-scFv clone, 2) develop a "prototype" chip to compare the expression level of a large amount of cell surface proteins using a 96-well plate format.

I have successfully developed the competitive binding condition as I have originally proposed. The detail is as follows: Three individual phage clones from a scFv antibody phage library, which have already been characterized to bear different range of binding ability to immunogen KLH, were used as my test case in evaluating competitive binding between HRP-conjugated rabbit IgG to mouse heavy plus light chain and KLH to the scFv displayed by phage. Several methods have been tried to immobilize phage in order to obtain the best orientation to expose scFv fusion protein. The following mode seems promising for my proposal. I coated each well of 96-well plate with 100ul of 2.5ug/ml anti-M13-antibody at 4°C overnight. Following block non-specific binding sites with 30mg/ml BSA at room temperature for 1 hour, 0.1nM of PEG8000 purified phage from three aforementioned representative clones was added in triplicate to indicating well to allow phage captured by anti-M13 antibody. On the side, the same amount of purified phage of one KLH negative clone was added in triplicate as control. After one-hour incubation and three stringent washings with TBS/0.05%Tween 20, a range of concentration of KLH from 0.4ug/ml to 50ug/ml was added to indicating well to bind the antigen binding sites of scFv displaying on the surface of phage. Following one-hour incubation and three thorough washings, 100ul of 5ug/ml HRP-conjugated rabbit IgG to mouse heavy plus light chain was added to each well to competitively bind to antigen binding site of scFv. After washing way the

unbound antibody and developing with OPD substrate about 5 minutes, the binding signal was measured in a microtiter plate reader at 492nm wavelength. The signal reversibly reflects the binding ability of an antigen to a scFv. The result (see figure1) shows this competitive binding condition is suitable to evaluate binding ability of an antigen to a scFv displaying by phage. This system is also ready to up-scale to evaluate the binding ability of a panel of plasma membrane proteins with a large amount of immobilized phage-scFv clones.

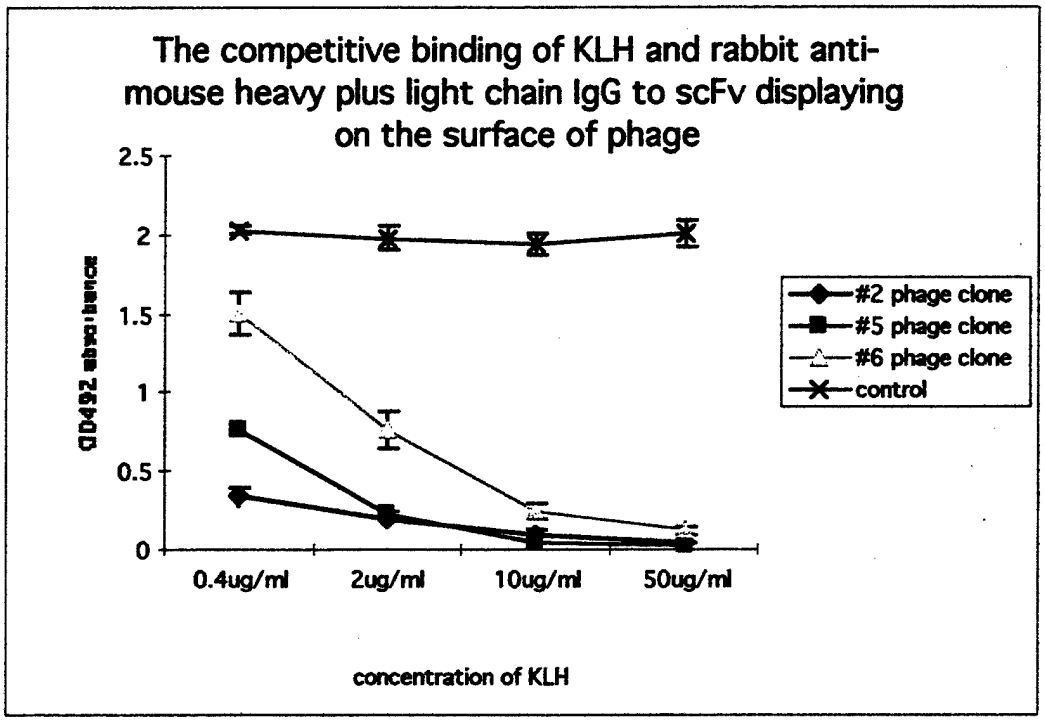

Figure1: Four phage-scFv clones have been used to test its binding ability to HRP-conjugated rabbit IgG to anti-mouse heavy chain plus light chain in the presence of various concentrations of KLH. According to my previous ELISA experiments: scFv displaying on the control phage does not recognize KLH; the arrange of binding ability of phage clone#2, #5 and #6 is clone#6>clone#5>clone#2. In this assay, the presence of KLH doesn't interfere the binding of control phage to rabbit anti-mouse heavy chain plus light chain IgG. But the bindings of phage clone#2, #5and #6 to rabbit anti-mouse heavy chain plus light chain IgG are partial blocked by KLH. Less and less rabbit IgG to anti-mouse heavy chain plus light chain is remained in the binding with the increasing concentration of KLH.

As I reported last year, eight unique phage clones out of seven hundred have been screened and demonstrated to recognize invasive breast carcinoma cell line specifically via ELISA and flow cytometry experiments. I amplified scFv cDNA from aforementioned eight phage clones and sub-cloned the fragment into PET-20b(+) vector to express soluble His-tagged scFv recombinant protein. Western Blot has been employed to confirm the expression of the His-tagged recombinant protein. Afterwards, I expressed His-scFv protein in large-scale, and purified fused protein using ProBond purification system. ProBond resin has a high affinity for the six tandem histidine residues. The fused His-scFv protein was eluted from ProBond resin by washing the resin with pH3.0 buffer. I have attempted to immunoprecipitate corresponding cell surface protein of invasive breast carcinoma cells by immobilizing the soluble recombinant scFv antibody on the nickel column, and passing cell lysate of MDA-MB-435 cells through the column. It will be desirable if I could obtain known cell surface antigen from MDA-MB-435 cell line as my positive control in the antibody chip assay. I failed in immunoprecipitating any target antigens from whole cell lysate. However, the binding feature of those eight phage clones to cell lysate of invasive breast carcinoma cell line provides the initial parameter for the development of a "prototype" chip in 96-well plate format, as well as provides a convenient first step toward scalp-up.

Key Research Accomplishments:

- I have constructed phage display scFv antibody libraries targeted to the plasma membrane proteins of invasive MDA-MB-435 and non-invasive MCF-7 breast carcinoma cell lines.
- I have succeeded in selecting a profile of phages displaying scFv antibodies to the cell surface proteins of MDA-MB-435 cells and MCF-7 by repeatedly panning phage library on intact cells.
- I developed the phage-cell ELISA assay. Seven hundred and five individual phage clones were tested for their binding ability to MDA-MB-435 cells with this ELISA assay. Forty-six of those clones showed specificity to the cell surface proteins on MDA-MB-435 cells.
- The specificity of ten of forty-six clones was confirmed by flow cytometry assay. The sequence analysis of those 10 clones showed that 8 clones have unique scFv antibody sequences.
- I expressed soluble scFv antibodies as recombinant fusion proteins with a His-Tag, so I am able to immunoprecipitate and identify the target protein of each antibody.
- I have developed a competitive binding condition that allows semi-quantification of the amount of antigen bound by immobilized individual phage-scFv clone.
- I have developed a "prototype" chip to compare the expression level of a large amount of cell surface proteins using a 96-well plate format.

Reportable Outcomes:

1. Harvey S, Zhang Y, Smith JW. Insights into a plasma membrane signature of mammary carcinoma. Physiological Genomics. 2001, 5(3): 129-136

CONCLUSIONS

The objectives of my study were:

- To develop an antibody chip for evaluating thousands of plasma membrane proteins (known or unknown) simultaneously.
- To understand the molecular mechanisms of metastasis by comparing of a collection of plasma membrane proteins of phenotypically distinct cell lines.

During the second award year of my fellowship, I have successfully developed a competitive binding condition to semi-quantify the amount of antigen bound by individual phage-scFv clone. This development bases on the rationale that the binding ability of rabbit anti-mouse heavy plus light chain antibody to the scFv fragment reversibly reflects the binding ability of antigen to the scFv antibody displaying by phage. This condition is also easy to up-scale to evaluate the binding ability of a panel of plasma membrane proteins to a large amount of immobilized phage-scFv clones simultaneously. Meanwhile, I have also well characterized the binding feature of eight distinct phage-scFv clones to cell lysate of invasive breast carcinoma cells, which provides the initial parameter for the development of a "prototype" chip in 96-well plate format. Both of the above mentioned achievements demonstrate it is possible to develop an high-density antibody chip to study thousands of plasma membrane proteins (known or unknown) of phenotypically distinct cell lines by combining phage display and micro-array technologies.

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